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The chemical warfare agent sulfur mustard (HD) produces a delayed inflammatory response followed by blister formation in skin of exposed individuals. There is a similarity between HD-induced skin injury and the skin disease Epidermolysis Bullosa (EB) in both the morphology of the damage and the structural components involved. Both HD-induced injury and EB are believed to involve matrix metalloproteinases (MMPs), which play key roles in the disruption of connective tissue proteins and basement membrane proteins. The objectives of this study are to examine HD-induced changes in gene expression of MMP-2, MMP-9, and their substrates (laminin-γ2, laminin-β3, and laminin5-α3A) in skin from mice cutaneously exposed to HD and to determine the efficacy of specific MMP inhibitors to protect against HD injury.

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INTRODUCTION

Sulfur mustard (bis(2-chloroethyl)sulfide, HD) is a chemical warfare agent that penetrates the skin rapidly and causes extensive blistering after a latent period of several hours (1-6). Currently, there is no established pharmacological countermeasure against HD-induced skin injury. Because the precise mechanisms responsible for HD-induced skin injury are unknown, treatment strategies and pharmacological countermeasures are under investigation. Epidermolysis bullosa (EB) is primarily a genetic human skin disease characterized by fragility and easy blistering of the skin in response to mechanical trauma. Based on skin pathology as well as analysis of the structural components involved, there appears to be a similarity between HD-induced skin injury and EB. It has been suggested that matrix metalloproteinases (MMPs) play a role in the structural damage incurred with EB. This study is divided into two sections: (1) a time course study and (2) a compound evaluation study. The specific aim of the time course study was to determine whether MMP and MMP substrate gene expression levels are altered over time (6, 12, 24, and 72 h) in mouse ear skin topically exposed to liquid HD. Gene expression profiles support the identification of specific targets for therapeutic countermeasures against HD-induced skin injury and provide additional biochemical markers for understanding HD toxicity. The specific aim of the compound evaluation study was to determine the effectiveness of topically delivered synthetic MMP inhibitors, Ilomastat, GM1489, MMP-2/MMP-9 Inhibitor I, and MMP-2/MMP-9 Inhibitor II, to protect against HD injury. Protection was quantitatively assessed by measuring MMP and MMP substrate gene expression levels with subsequent correlation to histopathological damage in tissues harvested at 24 h, 72 h and 7 days after HD challenge.

MATERIALS AND METHODS

HD and Compound Exposure

Male CD1 mice (Charles River Laboratories, Portage, MI; N = 20 per treatment) were anesthetized with ketamine and xylazine and exposed to HD and/or compound as previously described (7, 8). Briefly, five µL of 97.5 mM HD (0.08 mg) in CH_2Cl_2 (methylene chloride) was applied to the inner medial surface of the right ear. The left ear served as a control and received only the vehicle CH_2Cl_2. For the time course study, animals were euthanized at 6, 12, 24, and 72 h post-exposure and dermal punch specimens (8 mm in diameter) were taken from the center of both the HD-exposed and control ears. The ear punch was weighed to measure edema and then either snap-frozen in liquid nitrogen and stored at -70°C or fixed in neutral-buffered formalin for approximately 24 h at room temperature. After approximately 24 h the formalin-fixed tissues were rinsed in phosphate buffer saline (PBS) and stored at 4°C. The relative skin weight (RSW) between HD-treated and control groups was calculated [(weight of right ear - weight of left ear) / weight of left ear x 100] and statistical analyses were conducted using a one-way ANOVA (Statistica, StatSoft, Tulsa, OK). Statistical significance was defined as $P \leq 0.05$.

For the compound evaluation study, animals were divided into three treatment groups (Table 1). The compounds evaluated were Ilomastat, GM1489, MMP-2/MMP-9 Inhibitor I, and MMP-2/MMP-9 Inhibitor II (Calbiochem, SanDiego, CA). Group 1 received 5 µL of 97.5 mM HD (0.08 mg) in CH_2Cl_2 (methylene chloride) applied to the inner medial surface of the right ear and 5 µL of CH_2Cl_2 only applied to the left ear (left ear served as a control). Group 2 received 20 µL of 25 mM compound in ethanol applied to the right ear and 20 µL of ethanol only applied to the left ear (left ear served as a control). Group 2 was not exposed to HD. Group 3 received 20 µL of 25 mM compound in ethanol applied to the right ear and 20 µL of ethanol only applied to the left ear followed 15 minutes later with 5 µL HD applied to the right ear and 5 µL CH_2Cl_2-only applied to the left ear. At specified times (24 h, 72 h, and 7 days post-exposure) animals were euthanized and dermal punch specimens (8 mm in diameter) were taken from the center of both the right and left ears. The ear punch was weighed to measure edema and then either snap-frozen in liquid nitrogen and stored at -70°C

or fixed in neutral-buffered formalin for approximately 24 h at room temperature. The formalin-fixed tissues were rinsed in PBS and stored at 4°C.

Table 1. Treatment Groups for Compound Evaluation Study

Treatment Group	Right Ear	Left Ear
1	HD	CH_2Cl_2
2	Compound	Ethanol
3	Compound followed by HD	Ethanol followed by CH_2Cl_2

RNA Isolation and Reverse Transcription

Total RNA was isolated using TRIzol according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA) with the addition of PhaseLock Gel (Brinkman Eppendorf, Westbury, NY) during centrifugation to allow separation of the phenol-chloroform phase from the aqueous phase. The RNA pellet was dissolved in RNA Storage Solution (Ambion, Austin, TX). RNA was quantitated spectrophotometrically based on an absorbance at 260 nm of one equal to an RNA concentration of 40 μg/mL. Total RNA (1 μg) was reverse-transcribed into cDNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Gaithersburg, MD). A minus reverse transcriptase reaction was included as a control.

Real-Time Polymerase Chain Reaction (PCR)

Primer and probe sets for MMP-2, MMP-9, laminin-γ2, laminin5-α3A, and laminin-β3 were designed using the Assay-by-Design service of Applied Biosystems (Applied Biosystems, Foster City, CA) (Tables 2 and 3). Real-time PCR was performed an ABI Prism® 7900 HT Fast Real-Time-PCR Sequence Detection System. Hypoxanthine guanine phosphoribosyl transferase (HPRT) expression levels were used as an endogenous control. Three μL of cDNA was added to a 50 μL reaction. Assays were performed in duplicate and averaged. No template controls were negative for amplification. Threshold cycle (C*t*), which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increased above a threshold level.

The comparative C*t* method was used to determine relative mRNA expression levels for each of the test genes in the ear tissue samples. C*t* values for gene amplification were normalized by subtracting the C*t* values for HPRT using the equation: $Ct_{(gene)} - Ct_{(HPRT)} = \Delta Ct$. The ΔCt values for the control skin were subtracted from the HD-exposed skin ΔCt values to calculate the fold change in gene expression: $\Delta Ct_{(exposed)} - \Delta Ct_{(control)} = \Delta\Delta Ct$. Fold increases in gene expression were calculated by the following equation according to ABI User Bulletin #2: $2^{-\Delta\Delta Ct}$ = fold change in expression. Data is expressed as fold change.

Table 2. Gene Accession Numbers

Gene Name	Accession Number
Matrix metalloproteinase 2 (MMP-2)	NM_008610
Matrix metalloproteinase 9 (MMP-9)	Z27231
Laminin-γ2	U43327
Laminin-β3	NM_008484
Laminin-5α3A	X84013
Hypoxanthine guanine phosphoribosyl transferase (HPRT)	NM_013556

Table 3. Assay by Design Primer/Probe Sets

Forward Primer Name	**Forward Primer Sequence**
MMP2-529F	GCTGACATCATGATCAACTTTGGA
MMP9-997F	ACCAGGATAAACTGTATGGCTTCTG
Lamin-gam2-2306F	GCTCAGGAGGCTACAAGAAAGG
Lamin-5a3A-786F	AACAGATCCGGCACATGGA
Lamin-b3-2128F	GCAATTTGAGAAGCTAAGCAGTGA
MHPRT-485F	CAGTACAGCCCCAAAATGGTTAA
Reverse Primer Name	**Reverse Primer Sequence**
MMP2-529R	GCCATCAAACGGGTATCCAT
MMP9-997R	ACAGCTCTCCTGCCGAGTTG
Lamin-gam2-2306R	TGCTTCATTGCGTTAGCTGACT
Lamin-5a3A-786R	CCATGACTTGAGGTGGCAGAA
Lamin-b3-2128R	AGGACTGCTCATAAGCCATGGT
MHPRT-485R	AACACTTCGAGAGGTCCTTTTCAC
Probe Name	**Probe Sequence**
MMP2-529M1	CGCTGGGAGCATGG
MMP9-997M1	TACCCGAGTGGACGCG
Lamin-gam2-2306M2	AGCGTGGCTGTCTG
Lamin-5a3A-786M1	CCTGAGGAACCAGCTG
Lamin-b3-2128M1	CCTTCAGGAGCCTTC
MHPRT-485M2	CAGCAAGCTTGCAACC

RESULTS

Time Course of HD-Induced Skin Inflammation

Skin edema was determined at 6, 12, 24, and 72 h post-exposure and expressed as RSW (Table 4). A significant increase ($P \leq 0.05$) in RSW was observed in HD-treated skin over the observed time period. When compared to control skin weight, the mean HD-treated skin weight at each time period was significantly higher ($P <\leq 0.05$) than the respective controls. Edema was apparent within 12-24 h (Figure 1). At higher magnifications differences between the untreated (A and B) and treated samples were noted at 6 h post-treatment (Figure 2). By 12 h, an infiltration of inflammatory cells was observed in treated versus untreated samples (Figure 3). Damage to the epithelia increased with time and necrosis of basal cells and the appearance of subepidermal blisters was observed at 24 h (Figure 4) and 72 h (Figure 5) post-treatment. The 24 h and 72 h post-treatment samples also exhibited contralateral ear damage, secondary to the primary event (Figures 4 and 5). This was defined by the appearance of basal cell necrosis and subepidermal blisters on the untreated side of the ears (the outer ear).

Table 4. Relative Skin Weight for the Time Course Study

Time Post Exposure	Control Skin Weight (g)	HD-Treated Skin Weight (g)	RSW
6 h	0.0148	0.0190	29.1
12 h	0.0145	0.0340	134.9
24 h	0.0139	0.0372	168.5
72 h	0.0150	0.0458	207.1

Figure 1

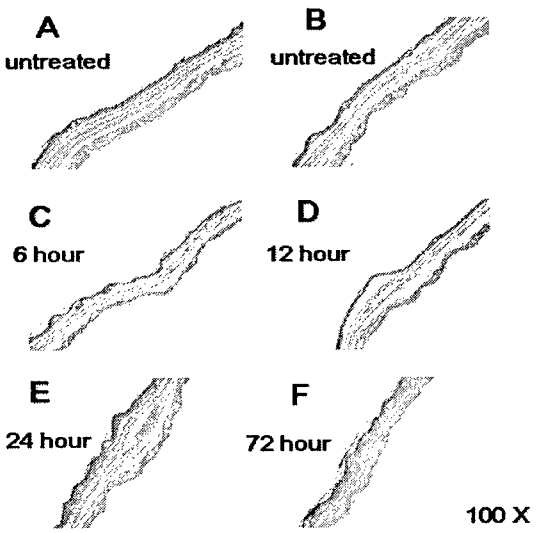

Figure 1. H & E staining from untreated (A and B) and HD-treated (C-F) mouse ear biopsy samples collected 6 (C), 12 (D), 24 (E), or 72 (F) h after HD exposure. The outer ear is toward the right and the inner ear toward the left. Magnification was 100X.

Figure 2

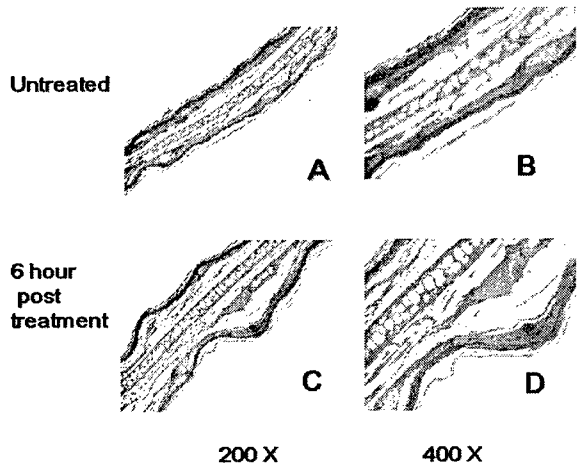

Figure 2. H & E staining from untreated (A and B) and 6 h post-HD-treated (C and D) mouse ear biopsy samples. Inner ear (treated) is to left and outer ear to right. Magnification either 200 X or 400X.

Figure 3

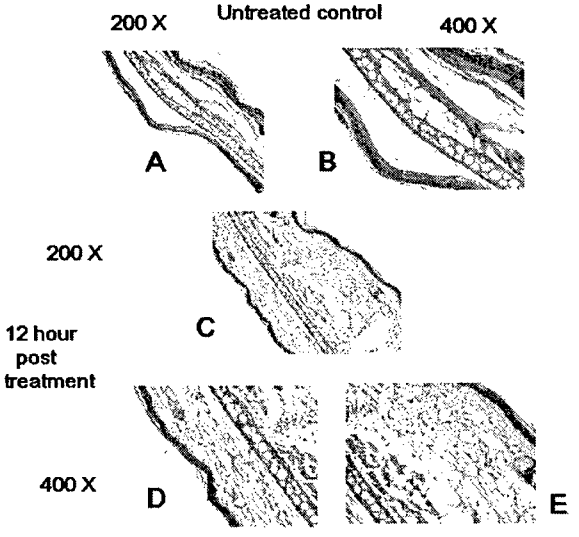

Figure 3. H & E staining from untreated (A and B) and 12 h post-HD-treated (C-E) mouse ear biopsy samples. Inner ear (treated) is to left and outer ear to right. Magnification either 200 X or 400X.

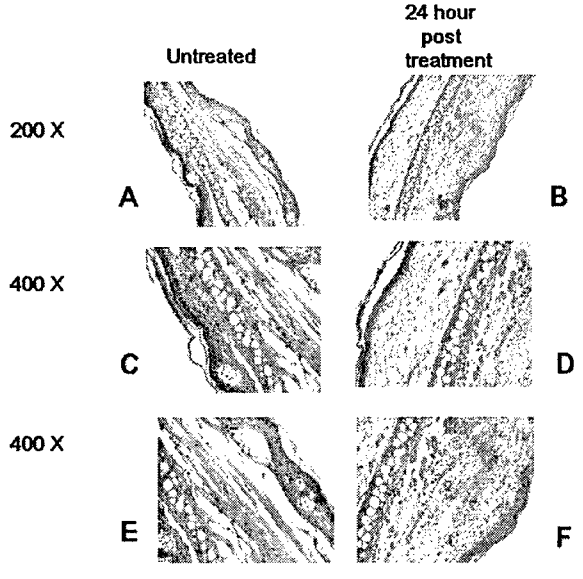

Figure 4. H & E staining from untreated (A, C, E) and 24 h post-HD-treated (B, D, F) mouse ear biopsy samples. Inner ear (treated) is to left and outer ear to right. Magnification either 200X or 400X.

Figure 5

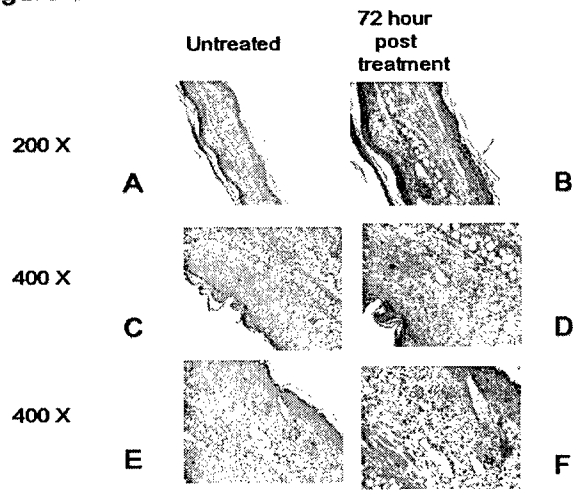

Figure 5. H & E staining from untreated (A, C, E) and 72 h post-HD-treated (B, D, F) mouse ear biopsy samples. Inner ear (treated) is to left and outer ear to right. Magnification either 200X or 400X.

Compound Evaluation Study

Skin edema was determined at 24 h, 72 h, and 7 days post-exposure and expressed as RSW (Table 5). Daily variation in ear tissue weights is normal when comparing weight changes between the HD-only and compound + HD treated groups. Because of these normal variations, statistics were not performed to determine if there was a significant difference in RSW between the HD-treated groups and the compound plus HD-treated groups at each time period. If a dramatic decrease had been observed, then further statistical analyses would be warranted. In the groups progressing to Day 7, the ear tissue became necrotic, and in many instances it was difficult to obtain a tissue specimen. Therefore, ear tissue weight at this time point is uninformative.

Table 5. Relative Skin Weight for Compound Evaluation Study

Compound	Time Post Exposure	RSW Compound + HD(g)	RSW HD-only(g)	RSW Compound only (g)
Ilomastat	24 h	146.2	138.4	0.1
	72 h	186.6	231.8	-2.7
	7 days	242.7	200.8	-3.0
GM1489	24 h	155.9	168.0	-0.7
	72 h	185.7	175.9	-1.8
	7 days	104.5	115.1	-1.4
MMP-2/ MMP-9 Inhibitor I	24 h	129.1	114.3	0
	72 h	154.1	124.6	-0.7
	7 days	173.5	160.4	-5.5
MMP-2/ MMP-9 Inhibitor II	24 h	148.0	154.5	0.9
	72 h	179.4	195.1	1.8
	7 days	161.0	146.4	0.5

To grade the extent of ear tissue damage, a modified Draize ear tissue scoring system was also conducted randomly at 24 h, 72 h, and 7 days. Scores range from 0 = unchanged from control tissue; 1 = edema and/or erythema; 2 = focal areas of necrosis, but tissue still pliable; 3 = less than 50% of tissue is necrotic, tissue firm and dry, and 4 = more than 50% of tissue is necrotic, tissue firm and dry. Scores at 24 h are all rated as "1", so no distinction between treatment groups can be made (Table 4). Scores at 7 days are reflective of mostly necrotic tissue (Table 6). There was a slight decrease in Draize scores when comparing HD treatment only to compound + HD treatment (Table 6).

Table 6. Draize Scores for Compound Evaluation Study

Compound	Time Post Exposure	Average Draize Scores Compound + HD	Average Draize Scores HD only
Ilomastat	24 h	1.0	1.0
	72 h	2.15	2.55
	7 days	3.9	3.95
GM1489	24 h	1.0	1.0
	72 h	2.2	2.6
	7 days	3.05	3.45
MMP-2/ MMP-9 Inhibitor I	24 h	1.0	1.0
	72 h	2.8	2.45
	7 days	3.7	3.8
MMP-2/ MMP-9 Inhibitor II	24 h	1.0	1.0
	72 h	2.5	2.45
	7 days	3.65	3.8

HD-Induced Gene Expression

PCR primer/probe sets were evaluated in real-time PCR using normal mouse ear tissue as the source of RNA for cDNA synthesis (Table 7). Each primer/probe set amplified its respective gene. The PCR primer/probes sets were then used to evaluate the gene expression levels of the genes in each of the samples from both the time-course study and the compound evaluation study.

Table 7. Evaluation of Primer/Probe Sets by Real-Time PCR with Normal Mouse Ear Tissue

Sample ID	Gene	C_T1	C_T2	C_T AVG
1-101-00-1	HPRT	25.39	25.43	25.69
1-101-00-2	HPRT	25.93	25.80	
1-102-00-1	HPRT	25.73	25.85	
1-101-00-1	MMP-2	19.04	19.11	19.21
1-101-00-2	MMP-2	19.18	19.21	
1-102-00-1	MMP-2	19.35	19.35	
1-101-00-1	MMP-9	25.33	NP	25.46
1-101-00-2	MMP-9	25.26	NP	
1-102-00-1	MMP-9	25.80	NP	
1-101-00-1	Laminin-γ2	27.04	NP	27.38
1-101-00-2	Laminin-γ2	27.59	NP	
1-102-00-1	Laminin-γ2	27.51	NP	
1-101-00-1	Laminin5-α3A	22.48	NP	22.60
1-101-00-2	Laminin5-α3A	22.75	NP	
1-102-00-1	Laminin5-α3A	22.56	NP	
1-101-00-1	Laminin-β3	23.16	NP	23.33
1-101-00-2	Laminin-β3	23.48	NP	
1-102-00-1	Laminin-β3	23.34	NP	

NP = Not Performed

For the time course study, gene expression levels were determined using real-time PCR in mouse ear tissue samples at 6, 12, 24, and 72 h post-exposure to HD. Data are presented graphically with geometric means and 95% confidence intervals (Figures 6 – 10) and in tabular form (Table 8). MMP-2 expression levels decreased from 6 to 24 h post-exposure and remained decreased at 72 h post-exposure (Figure 6). MMP-9 expression levels increased over the observed time period approximately 6-fold (Figure 7). Laminin-γ2 expression levels initially decreased at 6 and 12 h post-exposure followed by an increase at 24 h and 72 h post-exposure to approximately 6-fold (Figure 8). Laminin5-α3A expression levels were decreased at 6, 12, and 24 h post-exposure followed by an increase at 72 h post-

exposure (Figure 9). Laminin-β3 expression levels initially decreased at 6, 12, and 24 h post-exposure followed by a slight increase at 72 h (Figure 10).

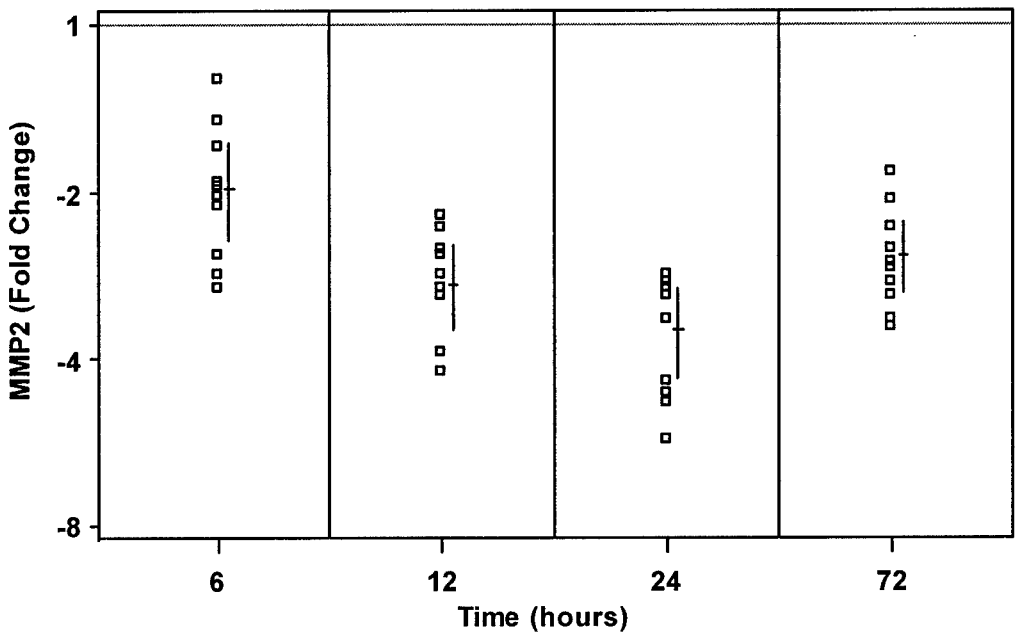

Figure 6. Fold Change in MMP-2 mRNA in Mouse Skin. Relative MMP-2 mRNA expression levels in mouse ear skin following a 0.08 mg HD cutaneous exposure compared to vehicle control at 6, 12, 24, and 72 h post-exposure. Individual data points are indicated by squares. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

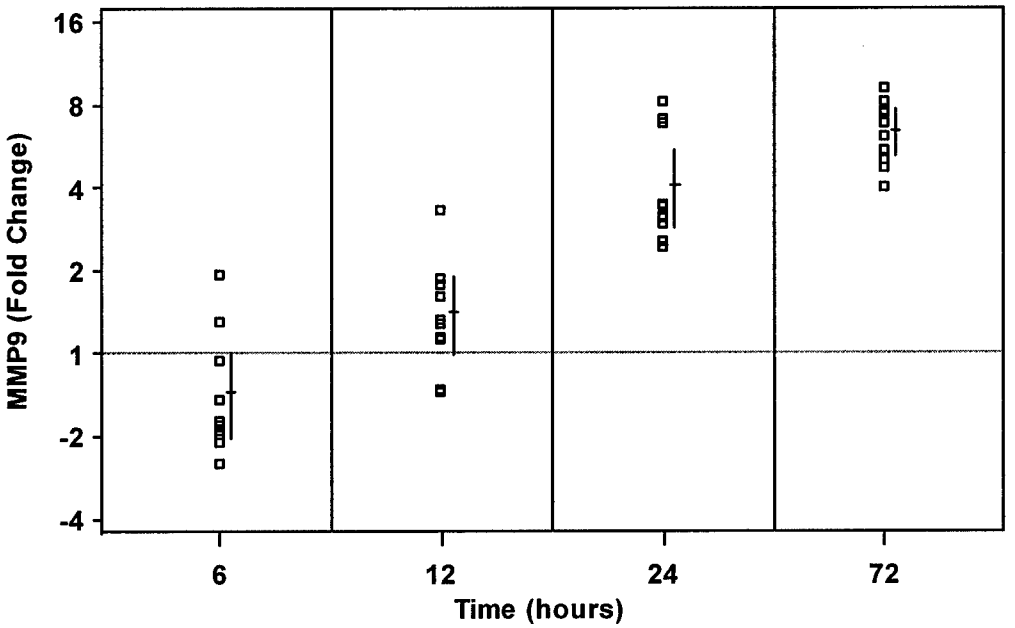

Figure 7. Fold Change in MMP-9 mRNA in Mouse Skin. Relative MMP-9 mRNA expression levels in mouse ear skin following a 0.08 mg HD cutaneous exposure compared to vehicle control at 6, 12, 24, and 72 h post-exposure. Individual data points are indicated by squares. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

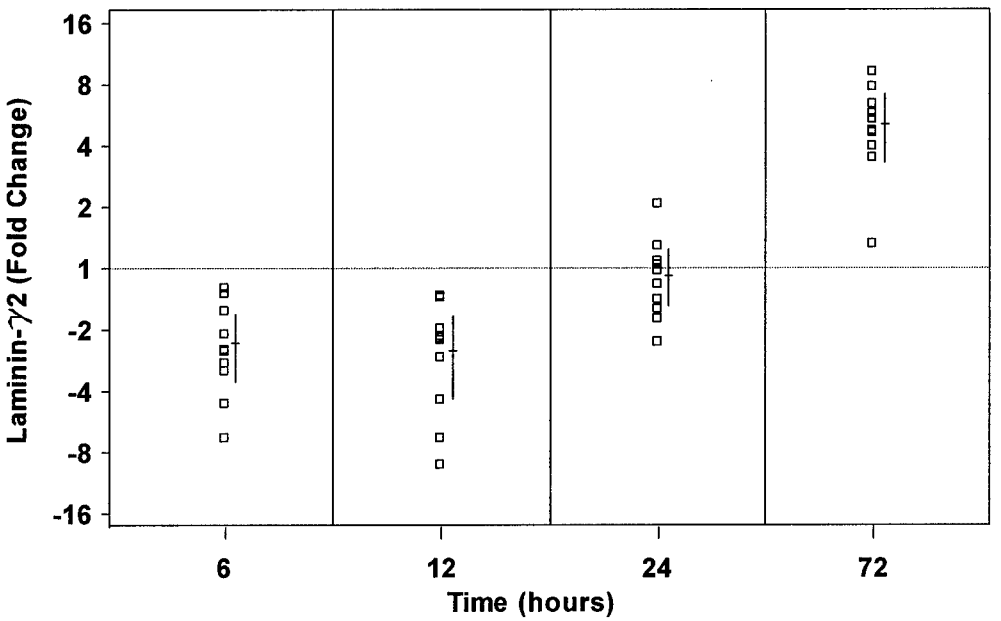

Figure 8. Fold Change in Laminin-γ2 mRNA in Mouse Skin. Relative laminin-γ2 mRNA expression levels in mouse ear skin following a 0.08 mg HD cutaneous exposure compared to vehicle control at 6, 12, 24, and 72 h post-exposure. Individual data points are indicated by squares. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

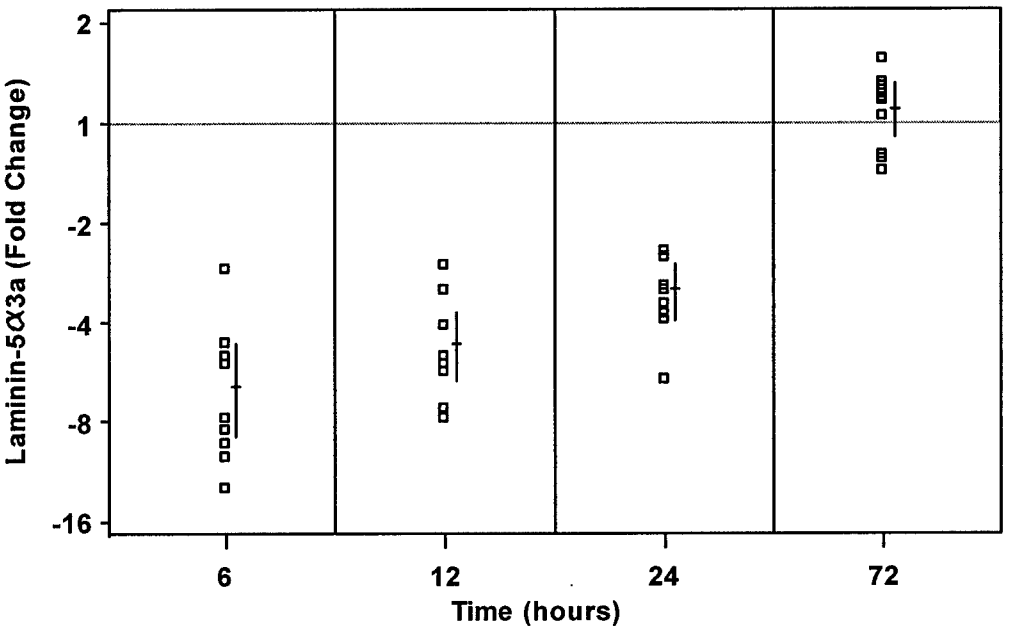

Figure 9. Fold Change in Laminin5-α3A mRNA in Mouse Skin. Relative laminin5-α3A mRNA expression levels in mouse ear skin following a 0.08 mg HD cutaneous exposure compared to vehicle control at 6, 12, 24, and 72 h post-exposure. Individual data points are indicated by squares. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

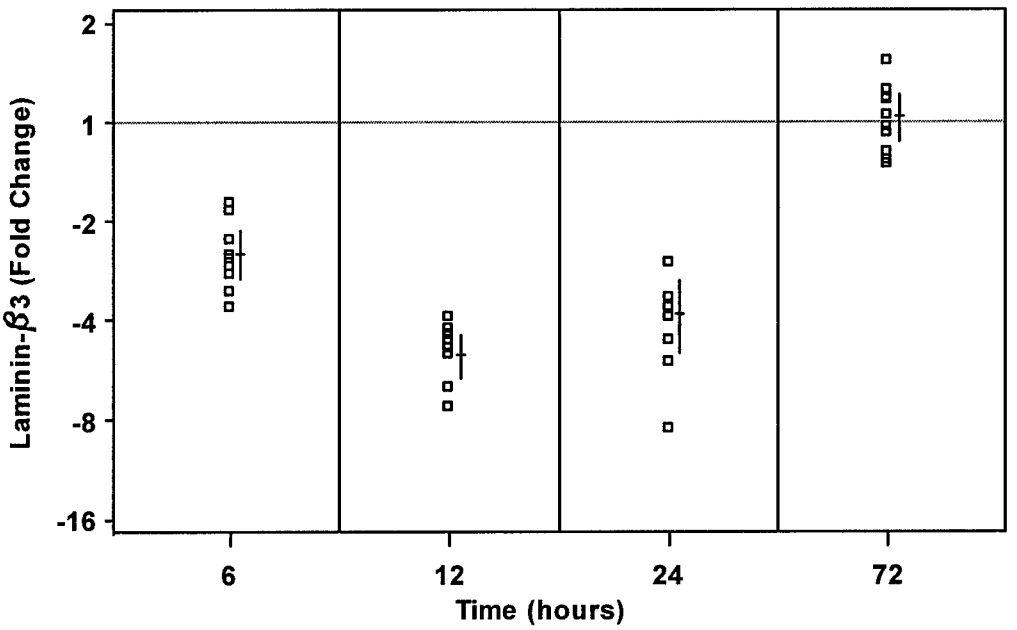

Figure 10. Fold Change in Laminin-β3 mRNA in Mouse Skin. Relative laminin-β3 mRNA expression levels in mouse ear skin following a 0.08 mg HD cutaneous exposure compared to vehicle control at 6, 12, 24, and 72 h post-exposure. Individual data points are indicated by squares. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

Table 8. Relative mRNA expression levels of MMP-2, MMP-9, laminin-γ2, laminin-5α3A, and laminin-β3 in mouse ear skin following a 0.08 mg HD cutaneous exposure compared to vehicle control at 6, 12, 24, and 72 h post-exposure.

Target Genes	Time (Hours)	HD Only		
		Fold Change (Geometric Mean)	95% Confidence Interval	
			Lower	Upper
MMP-2	6	-2.00	-2.44	-1.64
MMP-2	12	-2.94	-3.57	-2.50
MMP-2	24	-3.57	-4.35	-2.94
MMP-2	72	-2.63	-3.03	-2.27
MMP-9	6	-1.43	-2.04	1.00
MMP-9	12	1.37	-1.01	1.89
MMP-9	24	3.94	2.84	5.46
MMP-9	72	6.33	5.24	7.65
Laminin-γ2	6	-2.50	-3.57	-1.69
Laminin-γ2	12	-2.78	-4.35	-1.75
Laminin-γ2	24	-1.12	-1.54	1.23
Laminin-γ2	72	4.83	3.29	7.10
Laminin-5α3A	6	-6.25	-9.09	-4.55
Laminin-5α3A	12	-4.76	-5.88	-3.70
Laminin-5α3A	24	-3.23	-3.85	-2.63
Laminin-5α3A	72	1.09	-1.10	1.31
Laminin-β3	6	-2.50	-2.94	-2.13
Laminin -β3	12	-5.26	-5.88	-4.35
Laminin -β3	24	-3.85	-5.00	-3.03
Laminin -β3	72	1.03	-1.15	1.22

For the compound evaluation studies, gene expression analysis were performed for Ilomastat (Figures 11-15, Table 9), GM1489 (Figures 16-20, Table 10), MMP-2/MMP-9 Inhibitor I (Figures 21-25, Table 11), and MMP-2/MMP-9 Inhibitor II (Figures 26-30, Table 12). Data are presented graphically with geometric means and 95% confidence intervals (Figures 11-30) and in tabular form (Tables 9-12). Pre-treatment with Ilomastat in conjunction with HD exposure significantly decreased laminin-γ2 expression at 72 h and significantly increased laminin5-α3A expression at 72 h as compared to HD-only (no drug compound pre-treatment) (Tables 9 and 13). Pre-treatment with GM1489 in conjunction with HD exposure significantly decreased MMP-9 expression at 72 h and decreased MMP-2 expression at 7 days as compared to HD-only (Tables 10 and 13). Pre-treatment with MMP-2/MMP-9 Inhibitor I in conjunction with HD exposure significantly decreased MMP-2, laminin-γ2, laminin5-α3A, and laminin-β3 expression at 7 days and increased laminin-β3

and laminin5-α3A expression at 72 h as compared to HD-only (Tables 11 and 13). Pre-treatment with MMP-2/MMP-9 Inhibitor II in conjunction with HD exposure significantly increased laminin5-α3A expression at 24 h as compared to HD-only (Tables 12 and 13).

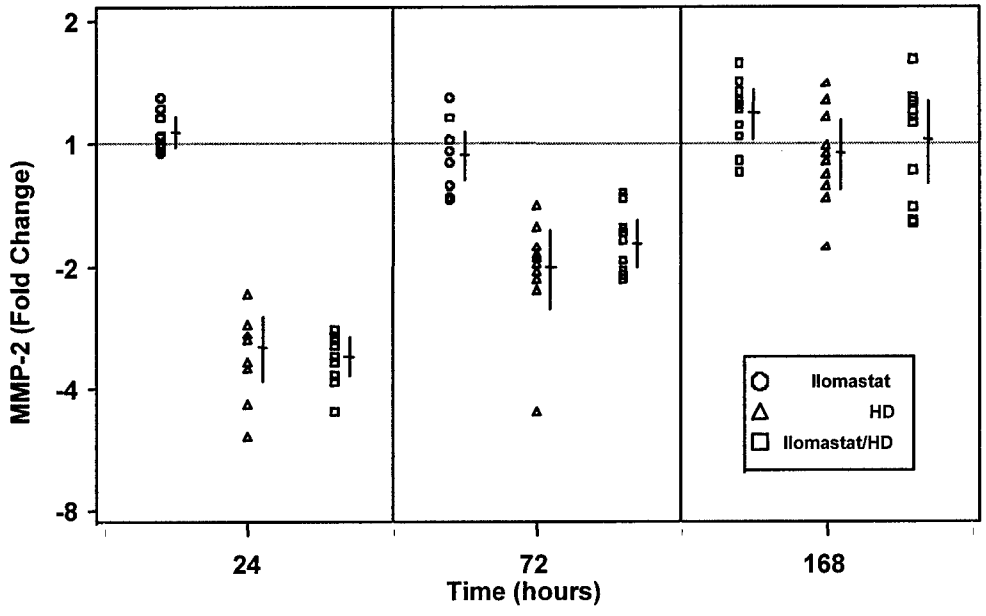

Figure 11. Effect of Ilomastat on MMP-2 mRNA Levels. Fold change in MMP-2 mRNA levels in mouse ear skin pretreated with Ilomastat only (circles), HD-exposed only (triangles), and pretreated with Ilomastat and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

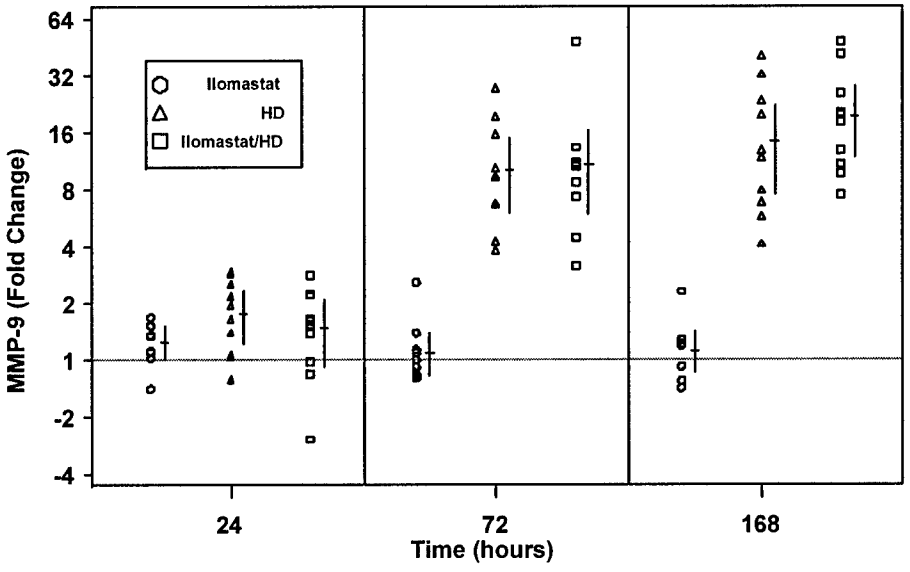

Figure 12. Effect of Ilomastat on MMP-9 mRNA Levels. Fold change in MMP-9 mRNA levels in mouse ear skin pretreated with Ilomastat only (circles), HD-exposed only (triangles), and pretreated with Ilomastat and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

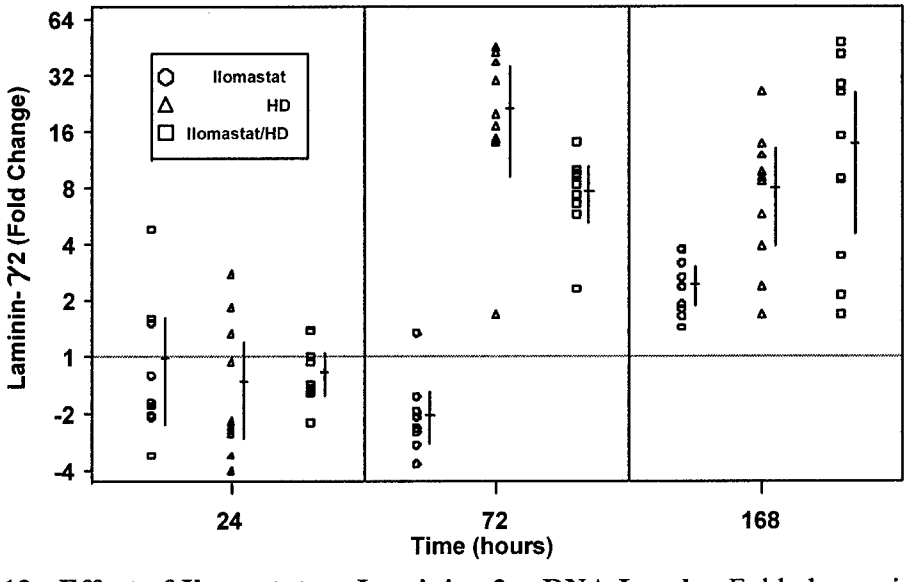

Figure 13. Effect of Ilomastat on Laminin-γ2 mRNA Levels. Fold change in laminin-γ2 mRNA levels in mouse ear skin pretreated with Ilomastat only (circles), HD-exposed only (triangles), and pretreated with Ilomastat and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

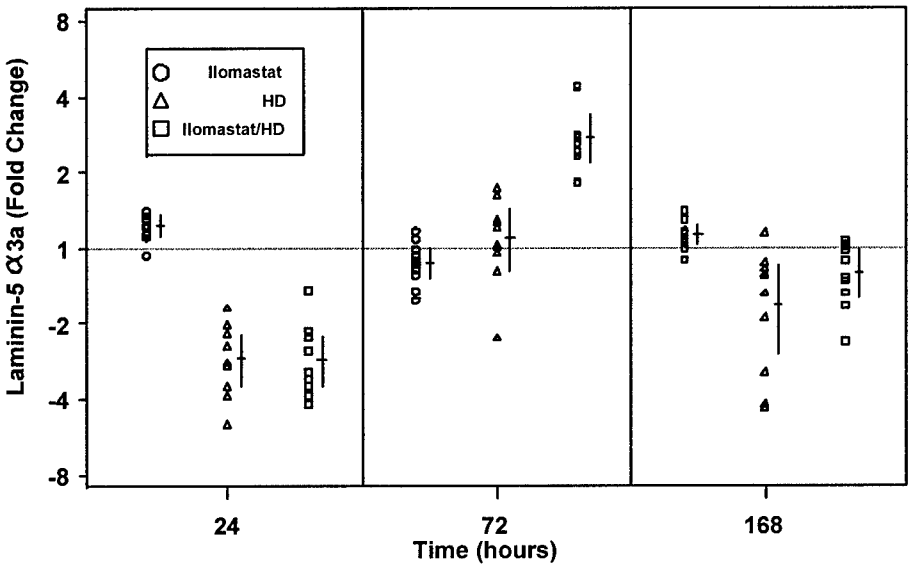

Figure 14. Effect of Ilomastat on Laminin-5α3A mRNA Levels. Fold change in laminin-5α3A mRNA levels in mouse ear skin pretreated with Ilomastat only (circles), HD-exposed only (triangles), and pretreated with Ilomastat and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

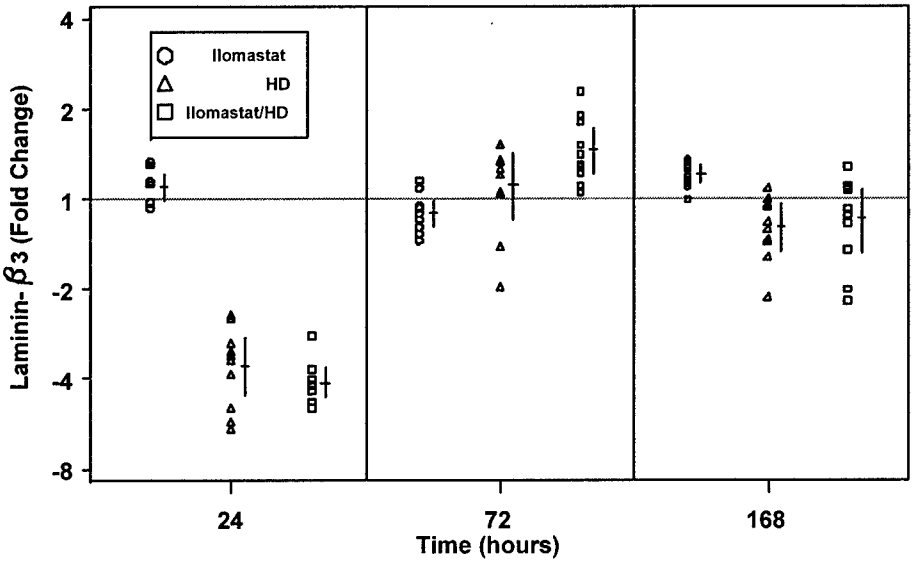

Figure 15. Effect of Ilomastat on Laminin-β3 mRNA Levels. Fold change in laminin-β3 mRNA levels in mouse ear skin pretreated with Ilomastat only (circles), HD-exposed only (triangles), and pretreated with Ilomastat and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

Table 9. Relative mRNA expression levels of MMP-2, MMP-9, laminin-γ2, laminin-5α3A, and laminin-β3 in mouse ear skin pretreated with Ilomastat only, HD-exposed only, and pretreated with Ilomastat and then HD-exposed at 24 h, 72 h, and 168 h post-exposure. Shaded values indicate significant difference between HD only and Ilomastat + HD based on Tukey's multiple comparisons test performed at an overall 0.05 significance level.

Target Genes	Time (Hours)	Ilomastat Only			HD Only			Ilomastat + HD		
		Fold Change (Geometric Mean)	95% Confidence Interval		Fold Change (Geometric Mean)	95% Confidence Interval		Fold Change (Geometric Mean)	95% Confidence Interval	
			Lower	Upper		Lower	Upper		Lower	Upper
MMP-2	24	1.07	-1.02	1.16	-3.23	-3.85	-2.63	-3.33	-3.70	-3.03
MMP-2	72	-1.08	-1.22	1.06	-2.04	-2.56	-1.64	-1.75	-2.00	-1.56
MMP-2	168	1.18	1.02	1.35	-1.08	-1.30	1.14	1.01	-1.25	1.27
MMP-9	24	1.22	-1.01	1.50	1.68	1.21	2.34	1.38	-1.10	2.09
MMP-9	72	1.06	-1.22	1.37	9.46	6.01	14.89	9.82	5.85	16.49
MMP-9	168	1.10	-1.18	1.41	12.86	7.42	22.29	18.18	11.74	28.15
Laminin-γ2	24	-1.19	-2.27	1.61	-1.52	-2.78	1.18	-1.25	-1.61	1.03
Laminin-γ2	72	-2.13	-2.86	-1.56	18.12	9.10	35.81	7.32	5.19	10.32
Laminin-γ2	168	2.36	1.85	3.00	7.07	3.88	12.88	10.86	4.54	26.00
Laminin-5α3A	24	1.23	1.11	1.36	-2.78	-3.57	-2.22	-2.86	-3.57	-2.27
Laminin-5α3A	72	-1.15	-1.33	1.00	1.07	-1.23	1.42	2.69	2.16	3.35
Laminin-5α3A	168	1.12	1.03	1.23	-1.79	-2.70	-1.18	-1.27	-1.59	-1.02
Laminin-β3	24	1.09	-1.02	1.21	-3.70	-4.55	-2.94	-4.17	-4.55	-3.70
Laminin-β3	72	-1.12	-1.25	-1.01	1.10	-1.18	1.42	1.45	1.21	1.74
Laminin-β3	168	1.21	1.13	1.29	-1.25	-1.49	-1.04	-1.19	-1.52	1.08

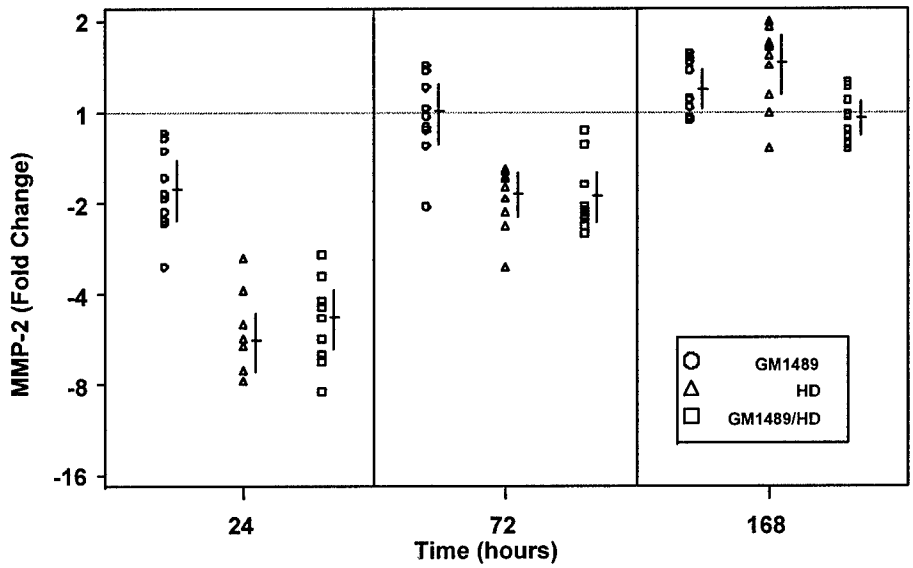

Figure 16. Effect of GM1489 on MMP-2 mRNA Levels. Fold change in MMP-2 mRNA levels in mouse ear skin pretreated with GM1489 only (circles), HD-exposed only (triangles), and pretreated with GM1489 and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

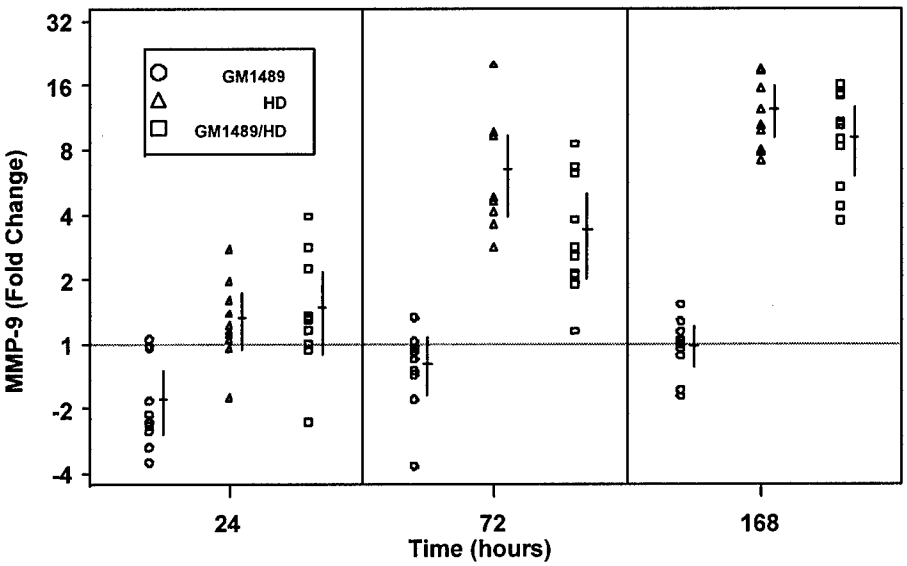

Figure 17. Effect of GM1489 on MMP-9 mRNA Levels. Fold change in MMP-9 mRNA levels in mouse ear skin pretreated with GM1489 only (circles), HD-exposed only (triangles), and pretreated with GM1489 and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

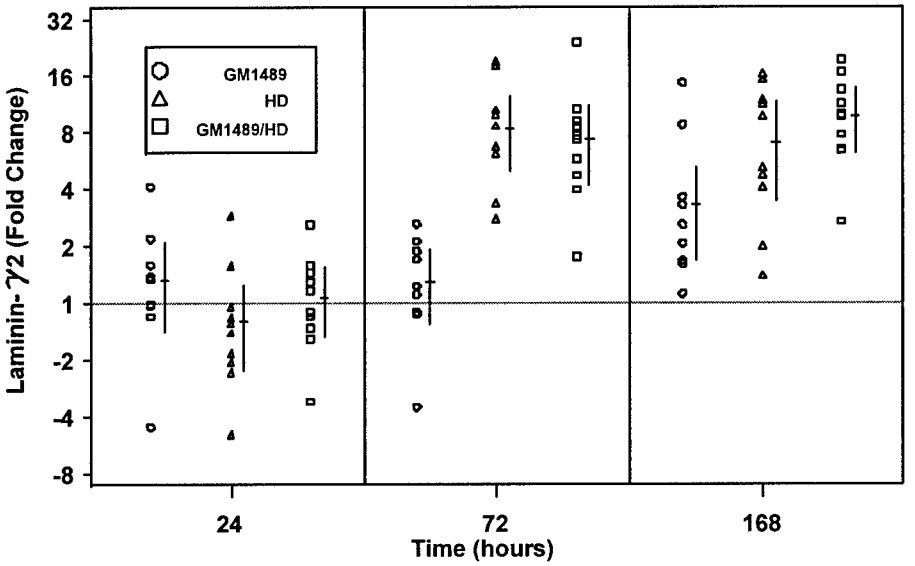

Figure 18. Effect of GM1489 on Laminin-γ2 mRNA Levels. Fold change in laminin-γ2 mRNA levels in mouse ear skin pretreated with GM1489 only (circles), HD-exposed only (triangles), and pretreated with GM1489 and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

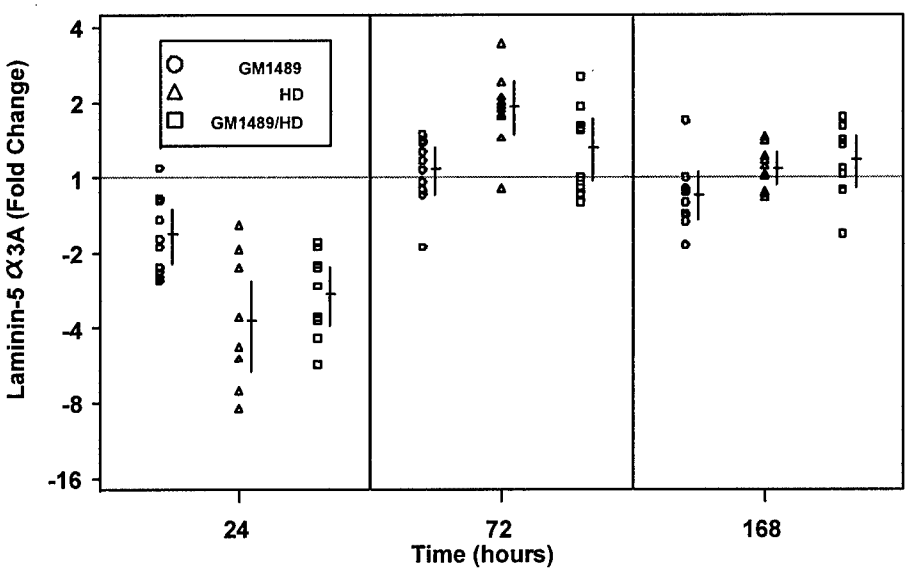

Figure 19. Effect of GM1489 on Laminin-5α3A mRNA Levels. Fold change in laminin-5α3A mRNA levels in mouse ear skin pretreated with GM1489 only (circles), HD-exposed only (triangles), and pretreated with GM1489 and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

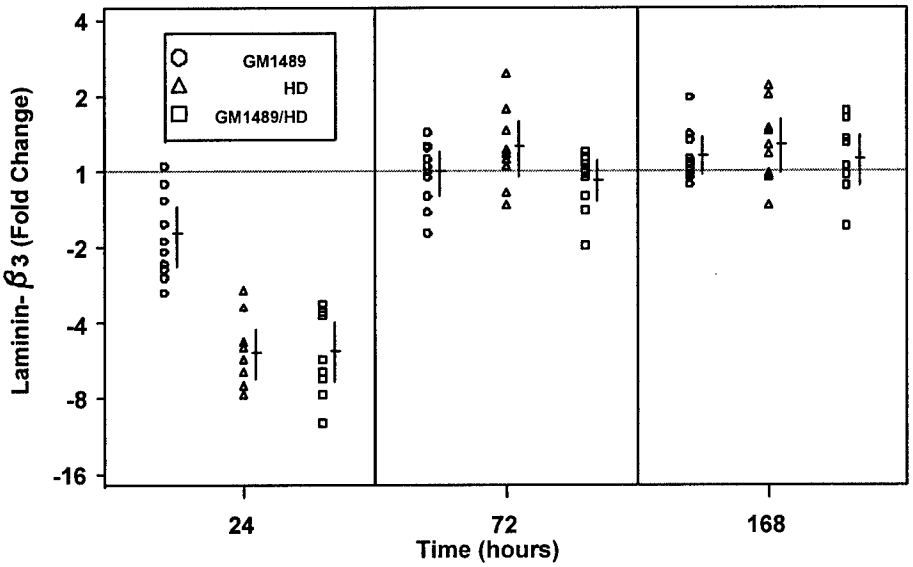

Figure 20. Effect of GM1489 on Laminin-β3 mRNA Levels. Fold change in laminin-β3 mRNA levels in mouse ear skin pretreated with GM1489 only (circles), HD-exposed only (triangles), and pretreated with GM1489 and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

Table 10. Relative mRNA expression levels of MMP-2, MMP-9, laminin-γ2, laminin-5α3A, and laminin-β3 in mouse ear skin pretreated with GM1489 only, HD-exposed only, and pretreated with GM1489 and then HD-exposed at 24 h, 72 h, and 168 h post-exposure. Shaded values indicate significant difference between HD only and GM1489 + HD based on Tukey's multiple comparisons test performed at an overall 0.05 significance level.

Target Genes	Time (Hours)	GM1489 Only			HD Only			GM1489 + HD		
		Fold Change (Geometric Mean)	95% Confidence Interval		Fold Change (Geometric Mean)	95% Confidence Interval		Fold Change (Geometric Mean)	95% Confidence Interval	
			Lower	Upper		Lower	Upper		Lower	Upper
MMP-2	24	-1.82	-2.27	-1.45	-5.88	-7.14	-4.55	-4.76	-6.25	-3.85
MMP-2	72	-1.01	-1.27	1.25	-1.89	-2.22	-1.59	-1.92	-2.33	-1.59
MMP-2	168	1.19	1.03	1.38	1.43	1.15	1.78	-1.05	-1.19	1.08
MMP-9	24	-1.89	-2.70	-1.35	1.27	-1.08	1.73	1.39	-1.12	2.18
MMP-9	72	-1.28	-1.75	1.06	6.06	3.92	9.36	3.15	1.99	4.99
MMP-9	168	-1.04	-1.28	1.20	12.02	9.09	15.90	8.76	6.05	12.68
Laminin-γ2	24	1.21	-1.43	2.07	-1.37	-2.27	1.24	1.00	-1.52	1.52
Laminin-γ2	72	1.21	-1.30	1.89	7.81	4.96	12.28	6.81	4.18	11.11
Laminin-γ2	168	2.95	1.67	5.22	6.25	3.40	11.50	9.16	6.09	13.78
Laminin-5α3A	24	-1.72	-2.22	-1.33	-4.00	-5.88	-2.56	-2.94	-3.85	-2.27
Laminin-5α3A	72	1.07	-1.18	1.33	1.89	1.47	2.41	1.29	-1.03	1.71
Laminin-5α3A	168	-1.19	-1.47	1.05	1.09	-1.06	1.25	1.16	-1.10	1.46
Laminin-β3	24	-1.82	-2.38	-1.37	-5.26	-6.67	-4.17	-5.26	-7.14	-4.00
Laminin-β3	72	-1.02	-1.25	1.21	1.23	-1.05	1.58	-1.09	-1.32	1.11
Laminin-β3	168	1.16	-1.03	1.37	1.26	-1.02	1.61	1.10	-1.14	1.38

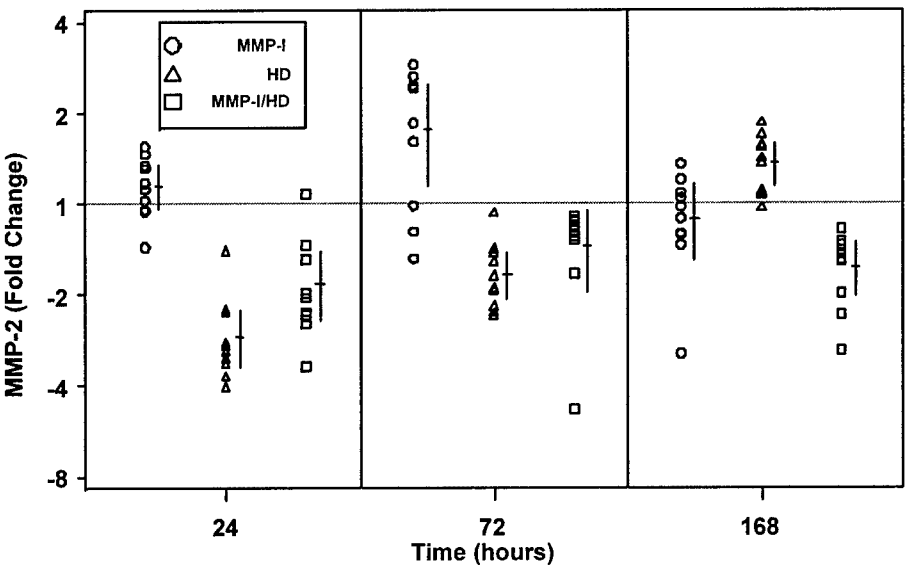

Figure 21. Effect of MMP-2/MMP-9 Inhibitor I (Inhibitor I) on MMP-2 mRNA Levels. Fold change in MMP-2 mRNA levels in mouse ear skin pretreated with Inhibitor I only (circles), HD-exposed only (triangles), and pretreated with Inhibitor I and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

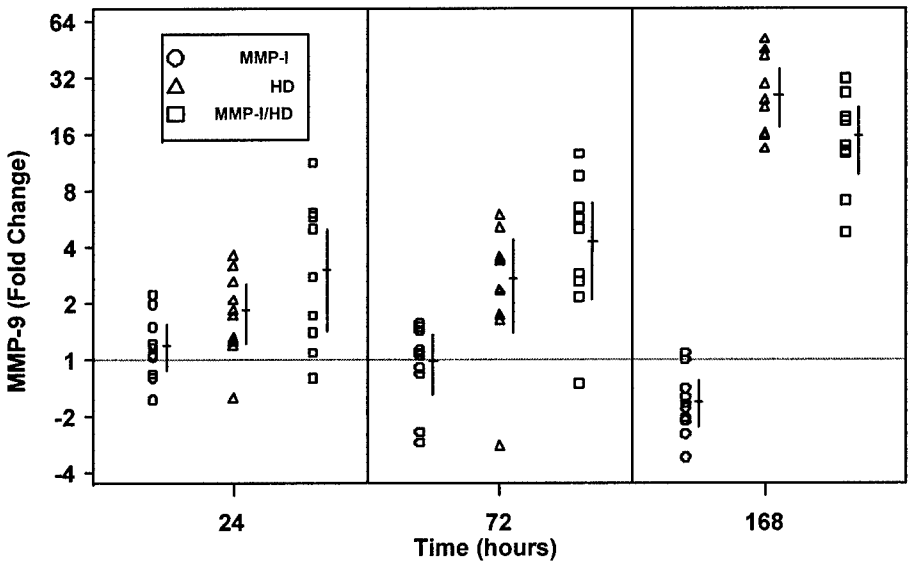

Figure 22. Effect of MMP-2/MMP-9 Inhibitor I (Inhibitor I) on MMP-9 mRNA Levels. Fold change in MMP-9 mRNA levels in mouse ear skin pretreated with Inhibitor I only (circles), HD-exposed only (triangles), and pretreated with Inhibitor I and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

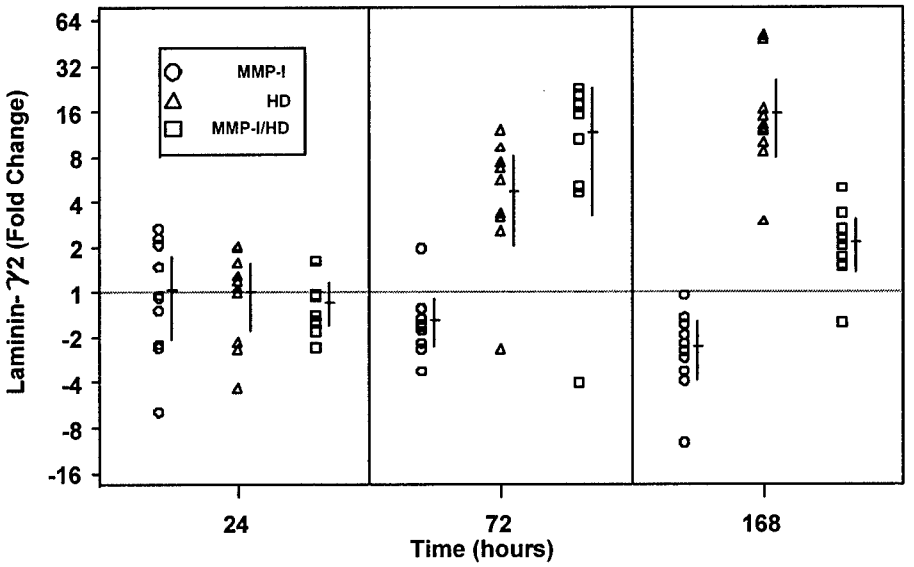

Figure 23. Effect of MMP-2/MMP-9 Inhibitor I (Inhibitor I) on Laminin-γ2 mRNA Levels. Fold change in laminin-γ2 mRNA levels in mouse ear skin pretreated with Inhibitor I only (circles), HD-exposed only (triangles), and pretreated with Inhibitor I and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

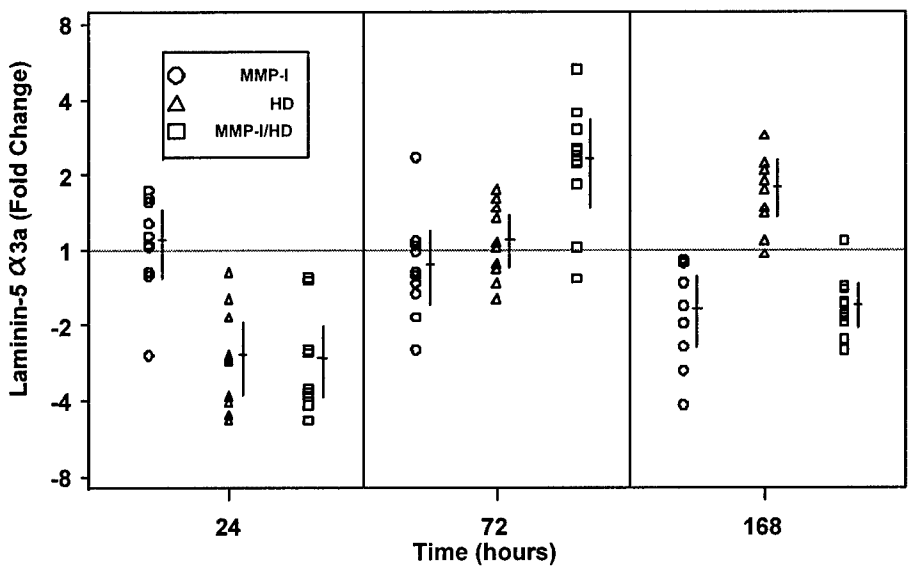

Figure 24. Effect of MMP-2/MMP-9 Inhibitor I (Inhibitor I) on Laminin-5α3A mRNA Levels. Fold change in laminin-5α3A mRNA levels in mouse ear skin pretreated with Inhibitor I only (circles), HD-exposed only (triangles), and pretreated with Inhibitor I and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

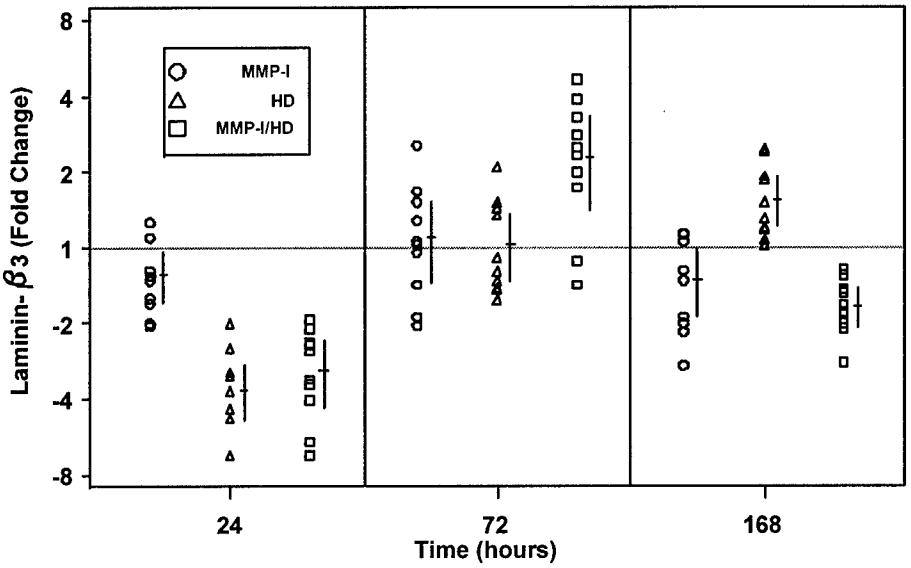

Figure 25. Effect of MMP-2/MMP-9 Inhibitor I (Inhibitor I) on Laminin-β3 mRNA Levels. Fold change in laminin-β3 mRNA levels in mouse ear skin pretreated with Inhibitor I only (circles), HD-exposed only (triangles), and pretreated with Inhibitor I and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

Table 11. Relative mRNA expression levels of MMP-2, MMP-9, laminin-γ2, laminin-5α3A, and laminin-β3 in mouse ear skin pretreated with MMP-2/MMP-9 Inhibitor I (Inhibitor I) only, HD-exposed only, and pretreated with Inhibitor I and then HD-exposed at 24 h, 72 h, and 168 h post-exposure. Shaded values indicate significant difference between HD only and Inhibitor I + HD based on Tukey's multiple comparisons test performed at an overall 0.05 significance level.

Target Genes	Time (Hours)	Inhibitor I Only			HD Only			Inhibitor I + HD		
		Fold Change (Geometric Mean)	95% Confidence Interval		Fold Change (Geometric Mean)	95% Confidence Interval		Fold Change (Geometric Mean)	95% Confidence Interval	
			Lower	Upper		Lower	Upper		Lower	Upper
MMP-2	24	1.14	-1.04	1.35	-2.78	-3.45	-2.27	-1.89	-2.44	-1.43
MMP-2	72	1.68	1.14	2.48	-1.72	-2.08	-1.45	-1.43	-1.96	-1.04
MMP-2	168	-1.15	-1.54	1.16	1.35	1.15	1.59	-1.64	-2.04	-1.35
MMP-9	24	1.15	-1.16	1.53	1.74	1.20	2.53	2.64	1.40	4.99
MMP-9	72	-1.08	-1.54	1.34	2.43	1.37	4.31	3.78	2.08	6.90
MMP-9	168	-1.72	-2.27	-1.30	24.93	17.53	35.44	14.71	9.74	22.23
Laminin-γ2	24	-1.10	-2.08	1.72	-1.08	-1.82	1.57	-1.20	-1.64	1.15
Laminin-γ2	72	-1.59	-2.27	-1.11	4.07	2.06	8.05	8.65	3.26	22.98
Laminin-γ2	168	-2.50	-3.85	-1.59	14.16	7.89	25.42	2.04	1.38	3.03
Laminin-5α3A	24	1.06	-1.32	1.46	-2.70	-3.85	-1.96	-2.78	-3.85	-2.00
Laminin-5α3A	72	-1.19	-1.67	1.19	1.08	-1.18	1.38	2.21	1.48	3.31
Laminin-5α3A	168	-1.79	-2.44	-1.28	1.76	1.35	2.30	-1.67	-2.04	-1.37
Laminin-β3	24	-1.32	-1.67	-1.03	-3.70	-4.76	-2.94	-3.23	-4.35	-2.33
Laminin-β3	72	1.05	-1.37	1.52	1.00	-1.35	1.35	2.15	1.40	3.32
Laminin-β3	168	-1.39	-1.92	-1.02	1.52	1.21	1.92	-1.75	-2.08	-1.45

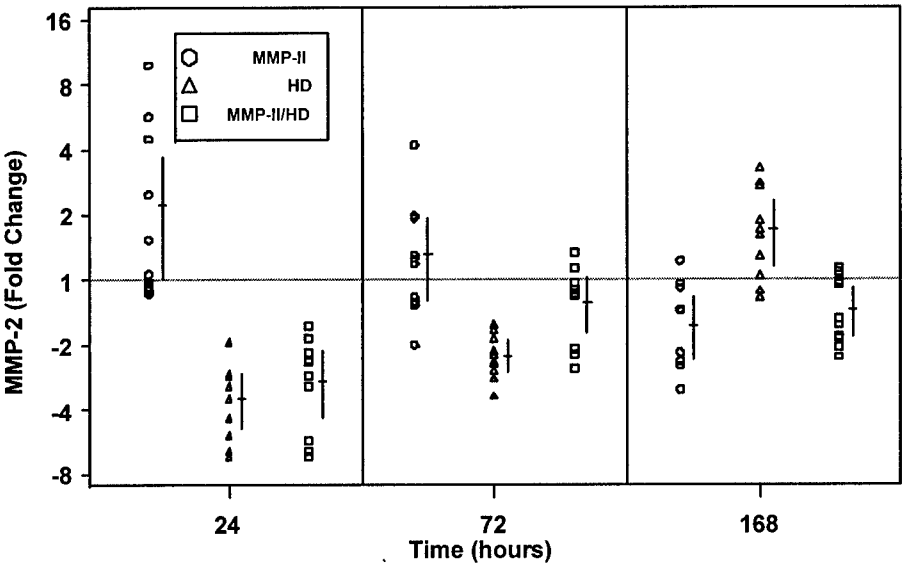

Figure 26. Effect of MMP-2/MMP-9 Inhibitor II (Inhibitor II) on MMP-2 mRNA Levels. Fold change in MMP-2 mRNA levels in mouse ear skin pretreated with Inhibitor II only (circles), HD-exposed only (triangles), and pretreated with Inhibitor II and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

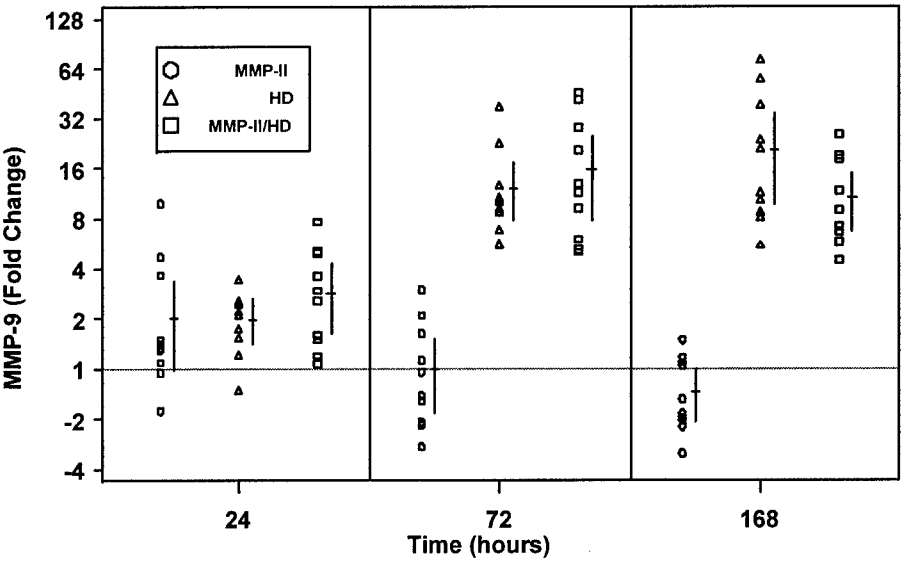

Figure 27. Effect of MMP-2/MMP-9 Inhibitor II (Inhibitor II) on MMP-9 mRNA Levels. Fold change in MMP-9 mRNA levels in mouse ear skin pretreated with Inhibitor II only (circles), HD-exposed only (triangles), and pretreated with Inhibitor II and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

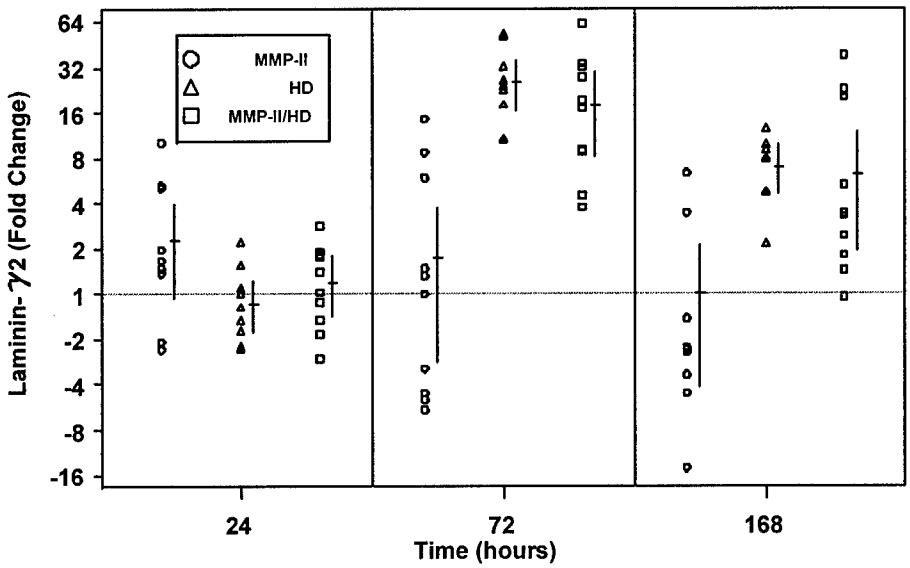

Figure 28. Effect of MMP-2/MMP-9 Inhibitor II (Inhibitor II) on Laminin-γ2 mRNA Levels. Fold change in laminin-γ2 mRNA levels in mouse ear skin pretreated with II only (circles), HD-exposed only (triangles), and pretreated with Inhibitor II and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

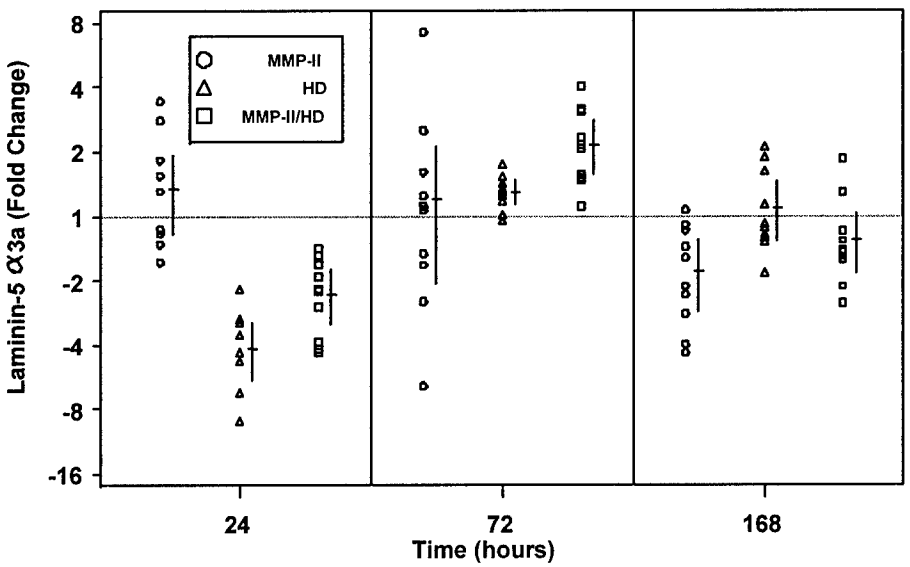

Figure 29. Effect of MMP-2/MMP-9 Inhibitor II (Inhibitor II) on Laminin-5α3A mRNA Levels. Fold change in laminin-5α3A mRNA levels in mouse ear skin pretreated with Inhibitor II only (circles), HD-exposed only (triangles), and pretreated with Inhibitor II and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

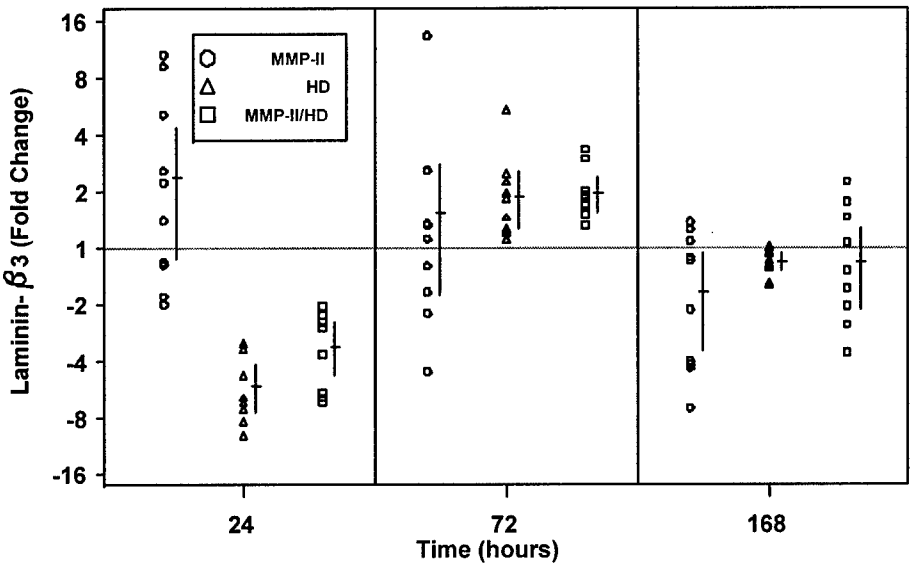

Figure 30. Effect of MMP-2/MMP-9 Inhibitor II (Inhibitor II) on Laminin-β3 mRNA Levels. Fold change in laminin-β3 mRNA levels in mouse ear skin pretreated with Inhibitor II only (circles), HD-exposed only (triangles), and pretreated with Inhibitor II and then HD-exposed (squares) at 24 h, 72 h, and 168 h post-exposure. Individual data points are indicated by the shapes. The horizontal dashes represent the geometric mean and the vertical lines represent the 95% confidence intervals.

Table 12. Relative mRNA expression levels of MMP-2, MMP-9, laminin-γ2, laminin-5α3A, and laminin-β3 in mouse ear skin pretreated with MMP-2/MMP-9 Inhibitor II (Inhibitor II) only, HD-exposed only, and pretreated with Inhibitor II and then HD-exposed at 24 h, 72 h, and 168 h post-exposure. Shaded values indicate significant difference between HD only and Inhibitor II + HD based on Tukey's multiple comparisons test performed at an overall 0.05 significance level.

Target Genes	Time (Hours)	Inhibitor II Only			HD Only			Inhibitor II + HD		
		Fold Change (Geometric Mean)	95% Confidence Interval		Fold Change (Geometric Mean)	95% Confidence Interval		Fold Change (Geometric Mean)	95% Confidence Interval	
			Lower	Upper		Lower	Upper		Lower	Upper
MMP-2	24	1.95	1.02	3.69	-3.70	-5.00	-2.70	-3.03	-4.35	-2.13
MMP-2	72	1.23	-1.27	1.90	-2.27	-2.70	-1.92	-1.32	-1.75	1.03
MMP-2	168	-1.69	-2.33	-1.20	1.63	1.14	2.31	-1.43	-1.82	-1.11
MMP-9	24	1.80	-1.02	3.34	1.91	1.39	2.62	2.65	1.63	4.31
MMP-9	72	-1.10	-1.85	1.51	11.48	7.70	17.11	13.84	7.64	25.07
MMP-9	168	-1.45	-2.08	-1.01	18.08	9.63	33.97	10.07	6.71	15.12
Laminin-γ2	24	1.91	-1.08	3.92	-1.20	-1.79	1.21	1.12	-1.41	1.78
Laminin-γ2	72	1.14	-2.86	3.70	24.01	16.28	35.41	15.66	8.11	30.25
Laminin-γ2	168	-1.41	-4.17	2.09	6.67	4.57	9.72	4.82	1.94	11.95
Laminin-5α3A	24	1.26	-1.22	1.92	-4.35	-5.88	-3.13	-2.38	-3.23	-1.79
Laminin-5α3A	72	1.01	-2.08	2.13	1.29	1.13	1.46	2.10	1.57	2.81
Laminin-5α3A	168	-1.92	-2.78	-1.30	1.05	-1.32	1.44	-1.33	-1.85	1.04
Laminin-β3	24	1.94	-1.15	4.35	-5.56	-7.69	-4.17	-3.45	-4.76	-2.50
Laminin -β3	72	1.24	-1.82	2.78	1.79	1.27	2.52	1.91	1.54	2.35
Laminin -β3	168	-1.92	-3.57	-1.06	-1.19	-1.33	-1.06	-1.30	-2.13	1.27

Table 13. Relative Gene Expression Levels Following Compound Pre-Treatment + HD as Compared to HD-Only

Compound	Time Point (h)	MMP-2	MMP-9	Laminin-γ2	Laminin5-α3A	Laminin-β3
Ilomastat	24	NC[1]	NC[1]	NC[1]	NC[1]	NC[1]
	72	NC[1]	NC[1]	↓	↑[3]	NC[1]
	168	NC[1]	NC[1]	NC[1]	NC[1]	NC[1]
GM1489	24	NC[1]	NC[1]	NC[1]	NC[1]	NC[1]
	72	NC[1]	↓	NC[1]	NC[1]	NC[1]
	168	↓[2]	NC[1]	NC[1]	NC[1]	NC[1]
MMP-2/MMP-9 Inhibitor I	24	NC[1]	NC[1]	NC[1]	NC[1]	NC[1]
	72	NC[1]	NC[1]	NC[1]	↑	↑
	168	↓	NC[1]	↓	↓	↓
MMP-2/MMP-9 Inhibitor II	24	NC[1]	NC[1]	NC[1]	↑	NC[1]
	72	NC[1]	NC[1]	NC[1]	NC[1]	NC[1]
	168	NC[1]	NC[1]	NC[1]	NC[1]	NC[1]

[1]NC = No change
[2]↓ = Significantly decreased gene expression
[3]↑ = Significantly increased gene expression

KEY RESEARCH ACCOMPLISHMENTS

- Mouse ear tissue has been collected and stored for both the initial time course study and the compound evaluation study.
- Gene expression analysis has been completed for the time course study and for the compound evaluation study.
- Gene expression analysis has been completed for the evaluation of Ilomastat, GM1489, MMP-2/MMP-9 Inhibitor I, and MMP-2/MMP-9 Inhibitor II pre-treatment.

REPORTABLE OUTCOMES

- A database of HD-induced alterations in the gene expression of MMP-2, MMP-9, laminin-γ2, laminin5-α3A, and laminin-β3 with and without compound pre-treatment

CONCLUSIONS

MMP-9 and laminin-γ2 mRNA levels increased in mouse skin over the 72 h examined in response to HD cutaneous exposure. MMP-2 mRNA levels decreased over the 72 h time period. Laminin5-α3A and laminin-β3 mRNA levels were decreased through 24 h, but increased above control levels at 72 h. In a previous study, MMP-9 mRNA levels

were elevated 2.5-fold in porcine skin at 24-72 h post-HD exposure (9). MMP-9 is likely involved in the pathology associated with HD-induced tissue injury; therefore, synthetic MMP inhibitors may be effective therapeutic agents against HD-induced tissue injury.

Based on our observations to date, candidate synthetic MMP inhibitors (Ilomastat, GM1489, MMP-2/MMP-9 Inhibitor I, and MMP-2/MMP-9 Inhibitor II) were selected for evaluation of their ability to protect against HD injury in the mouse ear skin model. Pre-treatment with Ilomastat in conjunction with HD exposure significantly decreased laminin-γ2 expression at 72 h and significantly increased laminin5-α3A expression at 72 h as compared to HD-only (no drug compound pre-treatment). This coincided with a slightly improved Draize Score at 72 h with Ilomastat pre-treatment as compared to the other compounds. Pre-treatment with GM1489 in conjunction with HD exposure significantly decreased MMP-9 expression at 72 h and decreased MMP-2 expression at 7 days as compared to HD-only. Pre-treatment with MMP-2/MMP-9 Inhibitor I in conjunction with HD exposure significantly decreased MMP-2, laminin-γ2, laminin5-α3A, and laminin-β3 expression at 7 days and increased laminin-β3 and laminin5-α3A expression at 72 h as compared to HD-only. Pre-treatment with MMP-2/MMP-9 Inhibitor II in conjunction with HD exposure significantly increased laminin5-α3A expression at 24 h as compared to HD-only.

This research addresses milestone objectives of the Joint Service Chemical and Biological Defense Program Defense Technology Objective (DTO) CB.30 (Medical Countermeasures for Vesicant Agents II). A primary objective of the DTO is to demonstrate safe and effective pharmacological countermeasures to prevent or decrease by 80% the severity of injuries caused by HD exposure. This study determined the *in vivo* efficacy of candidate protease inhibitor therapies in an animal model.

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